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Understanding the gut ecosystem: bugs, drugs & diseases

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MEDICAMENTO SUJETO
A PRESCRIPCIÓN MÉDICA
Mantener fuera del alcance
y de la vista de los niños
Leer el prospecto antes
de utilizar este medicamento
amoxicilina
clavulánico
Enantyum
comprimidos recubiertos
micralax citrato/

CHAPTER 8

Impact of 41 commonly used drugs on the composition, metabolic function and resistome of the gut microbiome

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Chapter 8

Abstract

The human gut microbiome is influenced by numerous factors including commonly used drugs, but it has also been shown that the gut microbiome by itself influences drug responses and efficacy. We studied the relations between commonly used drugs and microbial changes considering factors like polypharmacy and multi-morbidity.

We performed metagenomics sequencing of 1883 faecal samples from three cohorts: 1) a population-based cohort, 2) patients with inflammatory bowel diseases and 3) patients with irritable bowel syndrome. Differences between drug users and non-users were analysed per cohort, followed by a meta-analysis. 17 of 41 drugs were associated with changes in microbial features. For example, metformin users showed enrichment of *Escherichia-coli*-derived metabolic pathways and methanogenic pathways were increased in oral steroid users in patients with IBD.

Due the importance of the gut microbiome in health and the widespread use of drugs, we here provide evidence for extensive changes in taxonomy, metabolic activity and resistome in relation to commonly used drugs. Which paves the way to further mechanistic studies for drug response and side effects and has implications on future microbiome studies, underlining the need for correcting for multiple drug use in both general population and in GI disease cohorts.

Introduction

In recent years there has been growing interest in the associations between the gut microbial ecosystem and the use of non-antibiotic drugs. The interaction between drugs and gut microbe composition is important for understanding drug mechanisms and the development of certain drug side-effects[1,2].

The impact of antibiotics on gut microbiome composition has been well known for some time, but studies in population-based cohorts have found relations between multiple groups of drugs and gut microbiome signatures[3–6]. The use of proton pump inhibitors (PPIs) – drugs which inhibit stomach acid production – is associated with an increase in "typically oral" bacteria in the gut[7,8]. Metformin, a commonly used drug in type II diabetes has been associated with changes in gut microbiome composition both in vivo and in mice, in particular with an increase in bacteria that produce short chain fatty acids[9,10]. A recent study in a general population cohort showed that multiple drugs are associated with an altered gut microbiome composition[6]. In the same line, in vitro analysis of more than 1000 marketed drugs showed that non-antibiotic drugs can also inhibit the growth of common gut bacterial strains[11]. This, together with the fact that gut microbial composition has been linked to host conditions such as rheumatoid arthritis, inflammatory bowel disease (IBD) and susceptibility to enteric infections, suggests that some drug side-effects could be induced via their impact on the gut ecosystem[7,12–15].

To date, most of the studies published on this topic have focused on general population cohorts or single drug-microbe interactions[4–6]. However, these approaches do not reflect the clinical situation. Patients with gastrointestinal (GI) diseases like IBD and irritable bowel syndrome (IBS), for example, harbour a different gut microbiota composition than general population controls[15], and this could influence the occurrence of side effects or alter the mechanism of action of certain drugs. Moreover, patients with IBD or IBS also show differences in their patterns of drug-use compared to general population controls, including increased polypharmacy, either due to the GI disease itself or to other comorbidities[16–19]. In IBS, many commonly used drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) or antidepressants can trigger or alleviate GI symptoms[19]. Investigating drug-microbiome interactions could therefore lead to insights that can unravel the mechanisms involved in treatment response in IBD and the occurrence of GI symptoms with drug use in IBS.

To understand the impact of microbiome-drug interaction in humans, especially in the clinical context, it is crucial to consider the combination of different drug types. By meta-analysing the association between drug use and microbiome features in three independent cohorts from the same geographical region, we aimed to identify consistent relations as well as cohort-specific associations of commonly used drugs with gut microbiome composition. Through this data, we looked to pinpoint relevant changes on the microbial species and metabolic pathways and consequences for antibiotic resistance mechanisms in the gut in clinical context.

Results

Drug use

1126 of the 1883 participants from all cohorts were using at least one drug at time of faecal sampling. The number of drugs used per participant ranged from 0 to 12, with median values of 0 for the population cohort (mean 1.03, n=1124), 2 for the IBD cohort (mean 2.35, n=454) and 1 for the IBS cohort (mean 1.6, n=305) (Table 1, Supplementary table 1). In total, we observed 537 different combinations of drugs, with the most frequent being the combination of beta-sympathomimetic inhaler with steroid inhaler (18 users) (Supplementary Table 2). The use of steroid inhalers was strongly correlated with the use of beta sympathomimetic inhalers ($R_{population\text{-}cohort}$=0.78, $R_{IBD\text{-}cohort}$=0.65, $R_{IBS\text{-}cohort}$=0.78, p-value < 2×10^{-16}) (Supplementary Tables 3-5). In patients with IBD, the strongest correlation was observed between calcium and vitamin D supplements (R=0.84, p-value < 2×10^{-16}). Mesalazines (36%), thiopurines (33%) and anti-TNFα inhibitors (25%) were present in the top 10 most-used drugs in the IBD cohort. Since thiopurines and anti-TNFα inhibitors were solely used in the IBD cohort, these drugs were not included in multivariate analyses.

Microbial ecosystem and drug use

We first investigated the effect of each individual drug on the richness and overall gut microbial composition. As described earlier, disease cohorts presented a lower microbial richness compared to the general population cohort (Population cohort Shannon $Index_{mean}$= 2.26 (0.96-2.91), IBD cohort Shannon $Index_{mean}$=2.1 (0.38-2.78), IBS Shannon $Index_{mean}$=2.02 (1.01-2.65))[15]. Within cohorts, we did not observe any significant changes in the microbial richness associated with the use of any drug or in the number of different drugs used (Spearman correlation, p > 0.05, Supplementary table 6). However, we did observe differences between the number of drugs used on the overall microbial composition within all cohorts (Population cohort: r^2=0.006, FDR=0.001; IBD cohort: r^2=0.015, FDR=0.001; IBS cohort: r^2=0.014, FDR=0.0014, Supplementary table 7). PPIs were the only individual drug associated to compositional changes in all cohorts (Population cohort: r^2=0.006, FDR=0.001; IBD cohort: r^2=0.023, FDR=0.0006; IBS cohort: r^2=0.015, FDR=0.01).

Tab 1.

Drugs	LifeLinesDEEP (n=1124)	1000 IBD (n=454)	MIBS (n=305)
ACE inhibitors	44 (4 %)	24 (5 %)	7 (2 %)
Alpha blockers	10 (1 %)	3 (1 %)	7 (2 %)
AngII receptor antagonist	33 (3 %)	10 (3 %)	17 (6 %)
Anti androgen oral contraceptive	14 (1 %)	2 (0 %)	6 (2 %)
Anti epileptics	5 (0 %)	5 (1 %)	7 (2 %)
Anti histamine	69 (6 %)	15 (4 %)	14 (5 %)
Anti-TNFα	1 (0%)	119 (25 %)	0 (0%)
Antibiotics merged	13 (1 %)	12 (3 %)	7 (3 %)
Benzodiazepine derivatives related	25 (2 %)	16 (4 %)	13 (5 %)
Beta blockers	61 (5 %)	34 (8 %)	23 (8 %)
Beta sympathomimetic inhaler	64 (6%)	16 (4 %)	16 (6 %)
Bisphosphonates	10 (1 %)	13 (3 %)	4 (1%)
Ca-channel blocker	21 (2 %)	10 (2 %)	14 (5 %)
Calcium	14 (1 %)	76 (17 %)	8 (3 %)
Iron preparations	7 (1 %)	15 (3%)	1 (0 %)
Folic acid	7 (1 %)	31 (7 %)	0 (0 %)
Insulin	4 (0 %)	11 (2 %)	0 (0 %)
IUD that includes hormones	60 (5 %)	5 (1 %)	1 (0 %)
K-saving diuretic	7 (1 %)	9 (2 %)	1 (0 %)
Laxatives	21 (2 %)	30 (7 %)	27 (9 %)
Levothyroxine	26 (2 %)	10 (2 %)	5 (2 %)
Melatonine	6 (1 %)	4 (1 %)	1 (0 %)
Mesalazines	2 (0 %)	162 (36 %)	2 (1 %)
Metformin	15 (1 %)	7 (2 %)	6 (2 %)
Methylphenidate	6 (1 %)	5 (1 %)	1 (0 %)
NSAID	42 (4 %)	21 (5 %)	22 (7 %)
Opiat	13 (1 %)	22 (5 %)	7 (2%)
Oral anti diabetics	8 (1 %)	8 (2 %)	4 (1 %)
Oral contraceptive	113 (10 %)	55 (12 %)	32 (11 %)
Oral steroid	5 (0 %)	79 (17 %)	4 (1 %)
Other antidepressant	9 (1 %)	10 (2 %)	3 (1 %)
Paracetamol	11 (1 %)	42 (9 %)	42 (14 %)
Platelet aggregation inhibitor	32 (3 %)	27 (6 %)	18 (6 %)
PPI	93 (8 %)	108 (24 %)	48 (16 %)
SSRI antidepressant	28 (2 %)	10 (2 %)	30 (10 %)
Statin	55 (5 %)	28 (6 %)	26 (9 %)
Steroid inhaler	57 (5 %)	17 (4 %)	17 (6 %)
Steroid nose spray	55 (5 %)	6 (1 %)	7 (2 %)
Thiazide diuretic	43 (4 %)	17 (4 %)	17 (6 %)
Thiopurines	0 (0%)	151 (33 %)	0 (0%)
Tricyclic antidepressant	10 (1 %)	16 (4 %)	2 (1 %)
Triptans	20 (2 %)	5 (1 %)	2 (1 %)
Vitamin D	14 (1 %)	70 (15 %)	3 (1 %)
Vitamin K antagonist	5 (0 %)	7 (2 %)	6 (2 %)

Drug usage per cohort. Number and percentage of drug users in each cohort.

Associated taxa and pathways with drug use

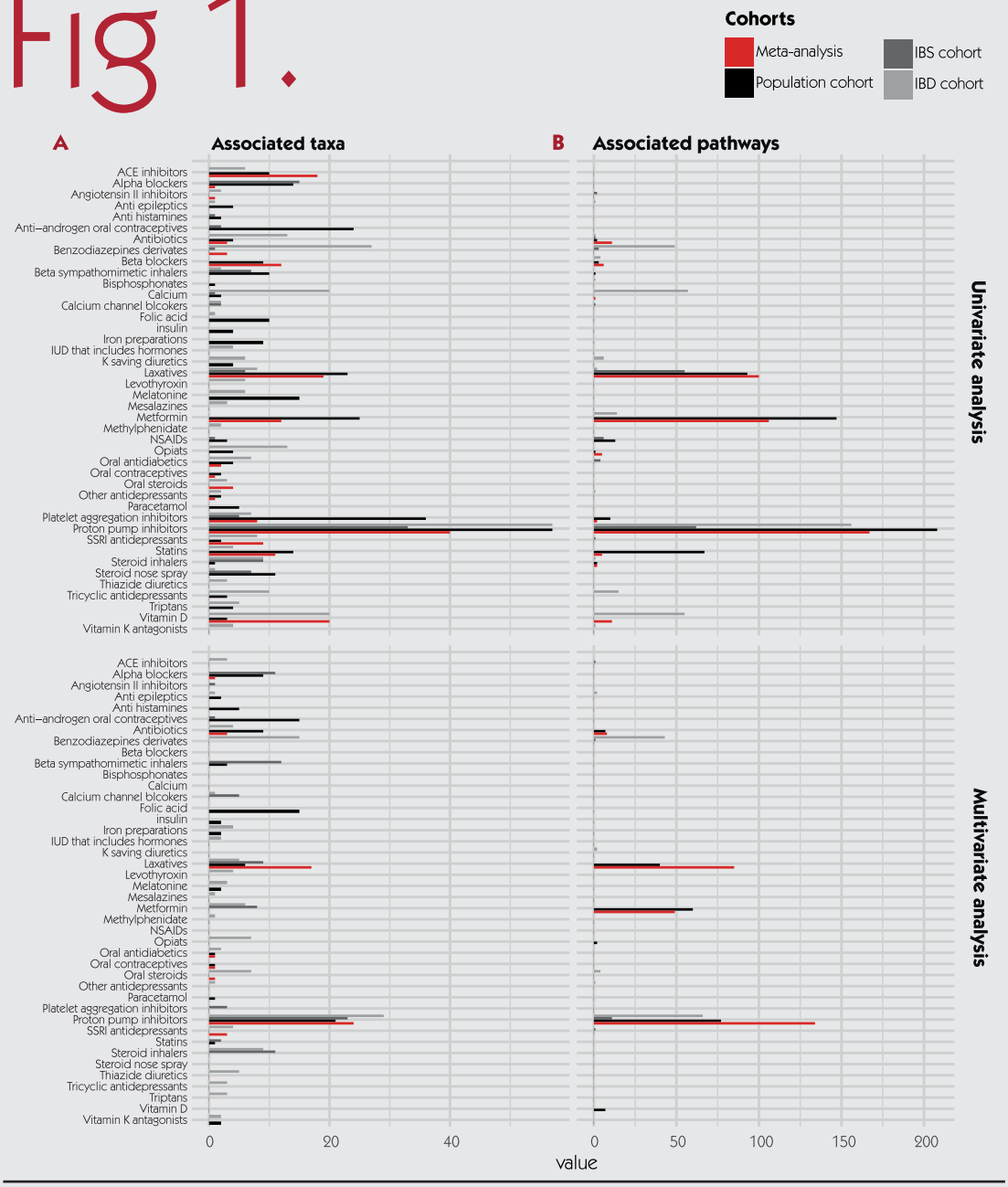
In the meta-analysis accounting for host age, sex and BMI, 154 associations between individual taxa and 17 groups of drugs were found to be statistically significant (FDR < 0.05) (Figure 1a, Supplementary table 8). PPIs, metformin, vitamin D supplements and laxatives were the individual drugs with the largest number of associations (> 10) in the univariate analysis. An interesting observation was that changes in the abundance of certain specific taxa were associated with multiple independent drugs. For example, the abundance of *Streptococcus salivarius* was increased in users of platelet aggregation inhibitors, opiates, SSRI antidepressants and vitamin D supplements. We did, however, also see features that were specific to individual drugs: an increased abundance of *Bifidobacterium dentium* was specific to PPI users (FDR=$9.38x10^{-7}$) and an increased abundance of *Eubacterium ramulus* was specific to participants using SSRI antidepressants (FDR=0.047). The use of drugs was also associated to functional changes in the gut. In the same analysis, 411 microbial pathways were related to 11 drugs (Figure 1b, Supplementary Table 9).

In order to consider multiple drug groups being prescribed at the same time, we tested each drug adding other drugs as covariates in the linear models. Overall, 46 associations were found between microbial relative abundance and the use of five drugs (Figure 1b, Supplementary Table 10). PPIs, laxatives and metformin showed the largest number of associations with microbial taxonomies and pathways. Despite the low number of antibiotics users, a decrease of *B. longum* abundance was observed in the population cohort (FDR=0.0046). Laxative users showed higher abundance of *Alistipes* and *Bacteroides* species in their microbiome. The association between SSRI antidepressant use and *Eubacterium ramulus* remained significant after correction for multi drug use (FDR=0.032). In the multivariate analysis, 273 pathways were associated to four drugs, including PPIs, laxatives, antibiotics and metformin (Supplementary Table 11). Interestingly, while the use of antibiotics was related to lower abundance of microbial pathways such as amino-acid biosynthesis, the use of metformin was associated with increased bacterial metabolic potential. Within the category of antibiotics, tetracyclines showed the strongest association with the altered pathways (Supplementary Table 12). Moreover, the abundance of microbial pathways in laxative- and PPI-users did show some similarities, including increase of glucose usage (increase of glycolysis and pathways) and decrease of starch degradation and aromatic compounds biosynthesis. All associations between taxonomy and pathways and individual drugs can be found in Supplementary table 13-46.

Cohort-specific changes in gut microbiome composition

Changes in the gut microbiota composition in patients with IBD and IBS have been observed before[15,20,21]. We therefore examined whether these changes were also present in the associations between the microbiota and the use of a drug. In the IBD cohort, benzodiazepine use was associated with an increased abundance of *Haemophilus parainfluenzae* (FDR=0.002, Supplementary table 47). Interestingly, this bacterium has also been described to be more prevalent in IBD patients than in healthy individuals. The use of tricyclic antidepressants was associated with an increased abundance of *Clostridium leptum* and

Fig 1.



Overview of the number of associated microbial features.

A) Bar-plot showing the number of associations between each type of drug and microbial taxa. The univariate model shows the association when considering one drug at the time while taking age, sex and BMI into account. The multivariate model considers the use of multiple drug types while taking age, sex and BMI into account. B) Bar-plot showing the number of associations between each drug type and microbial pathways.

intake of levothyroxine was associated with a higher abundance of *Actinomyces* (FDR=0.02 and 0.003, respectively). In addition, the 17 steroid inhaler users in the IBS cohort showed a higher abundance of *Streptococcus mutans* and *Bifidobacterium dentium* in their gut microbiome (FDR=0.001 and 0.01, respectively, Supplementary Table 40). Interestingly, patients with IBD using oral steroids showed a higher abundance of *Methanobrevibacter smithii* (FDR=0.002, Supplementary table 47). This association was also reflected at pathway level: the four pathways associated to the use of this drug also showed a high correlation with the abundance of *Methanobrevibacter smithii* (minimum rho coefficient=0.93, Supplementary table 48 and Supplementary figure 2). Two of these pathways are involved in methanogenesis, one in the biosynthesis of vitamin B2 and the last in the biosynthesis of nucleosides. Conversely, the use of other medication usually prescribed to treat IBD did not show strong associations with the microbial composition. Only the abundance of an *Erysipelotrichaceae* species was found to be slightly increased in mesalazine users (FDR=0.047, Supplementary table 49).

PPI use is related to increased function of upper-GI-tract bacteria

PPIs accounted for the largest number of associations, with a total of 40 altered taxa and 167 altered microbial pathways in the univariate analyses (Supplementary tables 8-9).

When correcting for the impact of other drug types, 24 taxa and 131 microbial pathways remained significantly associated with PPIs. We observed an increased abundance of *Veillonella parvula*, which is known to establish a mutualistic relation with *Streptococcus mutans* by co-aggregating and transforming the metabolic products of carbohydrate fermenting bacteria[22] (FDR=1.61×10^{-6} and 6.13×10^{-24}, respectively).

Functional changes included the increase of fatty acid and lipid biosynthesis, fermentation NAD metabolism and biosynthesis of L-arginine. The pathways associated with PPI-use involves functions that have a broad taxonomic contribution. However, a closer look at the predicted microbial contribution and at the gene families involved in each pathway revealed that the enrichment of specific microbial mechanisms is likely to be explained by the changes observed in taxonomical composition. Purine deoxyribonucleoside degradation, a pathway used as a source of energy and carbon, was predicted from the genomes of more than 25 different bacterial genera (figure 2). The increase in this function in the gut microbiome of PPI users can be explained by an increased abundance of *Streptococcus* species (*S.salivaris, S.parasanguinis* and *S.vestibularis*) (FDR<0.05). Three pathways involved in L-arginine biosynthesis (MetaCyc ID: PWY-7400, ARGSYNBSUB and ARGSYN) were more abundant in the microbiome of PPI users. While several bacterial taxa, including *Bifidobacterium* and *Ruminococcus* species, were predicted to contribute to these pathways, only *Streptococcus mutans* pathways showed a significant enrichment (FDR<0.05, Wilcoxon-test, Supplementary table 50). These analyses have also been performed in the medication's metformin, antibiotics and laxatives (Supplementary tables 51-53).

Different types of PPIs, namely omeprazole, esomeprazole and pantoprazole, exhibited a similar effect on the gut microbiome. Additionally, of the 131 microbial pathways associated with PPI use, 46 pathways also showed dosage dependent effects (FDR<0.05). For

example, participants using a higher dosage of PPIs (more or equal to 40 mg/day) showed a marked decrease in a pathway involved in the biosynthesis of amino acids (PWY-724) when comparing to low dosage users (FDR = 0.00064, Supplementary Table 12).

Metformin use is associated to changes in metabolic potential of enterobacteria

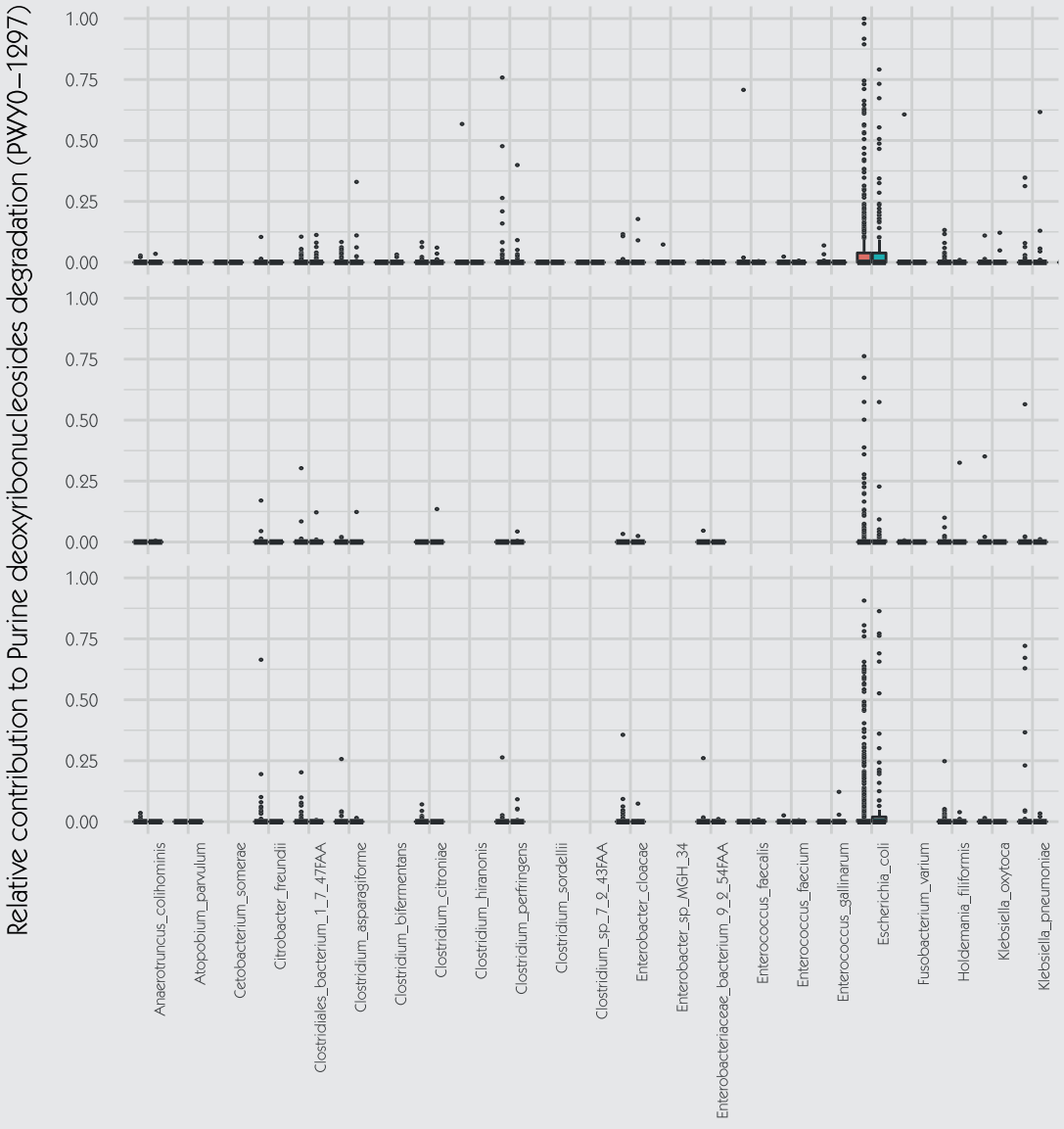
While changes in the abundance of *Streptococcus*, *Coprococcus* and *Escherichia* species were initially found to be enriched in metformin users, these associations were no longer significant when correcting for the use of other drug types. However, a suggestive association with *Escherichia coli* (FDR=0.08, Supplementary table 10) remained and, in the IBD cohort, the abundance of *Streptococcus mutans* was slightly increased in participants using this drug (FDR=0.05) (Supplementary table 47).

Strikingly, the functional implications of metformin use were large, even after correction for the use of other drugs, with 49 microbial pathways altered compared to the non-users. Metformin use was associated with changes in the metabolic potential of the microbiome, in particular with increases in the butanoate production, quinone biosynthesis, sugar derivatives degradation and polymyxin resistance pathways (FDR<0.05, Supplementary table 11). Interestingly, metagenomic pathways prediction and gene family analyses revealed that Enterobacteriaceae species, mainly *Escherichia coli*, were the major contributors to the functional changes associated with metformin use. Our data suggest that physiological changes induced by metformin can provide competitive advantage to enterobacterial species which could potentially have implications on health (Supplementary table 51). Furthermore, we did not identify dosage dependent effects on metformin usage on the associated pathways (Supplementary table 12).

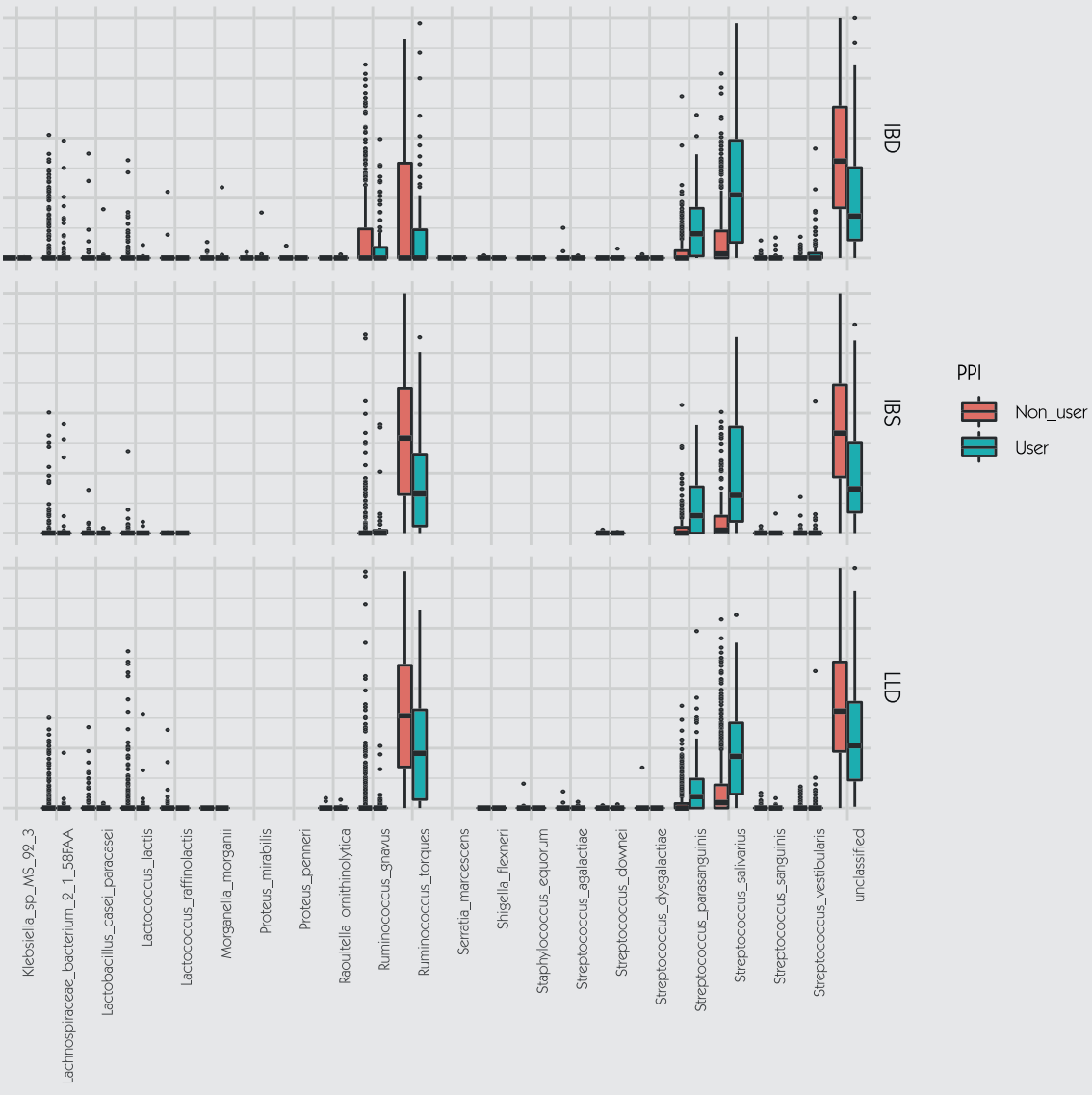
SSRI antidepressant use effect is evident in patients with IBS

SSRI antidepressants were among the top 10 most-used drugs in the IBS cohort. In users of this drug type, the only taxa that remained statistically significant in the multivariate meta-analysis was the increased abundance of *Eubacterium ramulus* (FDR=0.032). This medication category included six different subtypes of drugs in which paroxetine represented 32% of the SSRI-users (supplementary table 1). Interestingly, and despite the low numbers, the increased abundance of *Eubacterium ramulus* was mainly observed in the paroxetine users (FDR = 0.003, Supplementary table 12). Previous research has shown that dietary flavonoids also lead to an increase in this species[23]. Furthermore, the pathway involved in peptidoglycan maturation was decreased in the multivariate meta-analysis of SSRI antidepressant users compared to non-users (FDR=0.13). However, this finding was mainly observed in the IBS cohort (FDR=0.002, Supplementary Tables 38).

Fig 2.



Microbial contribution to the purine deoxyribonucleoside degradation pathway.
Box plots representing the relative contribution of each microbe to the overall pathway quantification. Blue box-plots represent the values of PPI users and red box-plots represent the values of non-PPI users. Each row represents one of the studied cohorts.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Lachnospiraceae_bacterium_2_1_58FAA
Lactobacillus_casei_paracasei
Lactococcus_lactis
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Morganella_morganii
Proteus_mirabilis
Proteus_penneri
Raoultella_ornithinolytica
Ruminococcus_gnavus
Ruminococcus_torques
Serratia_marcescens
Shigella_flexneri
Staphylococcus_equorum
Streptococcus_agalactiae
Streptococcus_downei
Streptococcus_dysgalactiae
Streptococcus_parasanguinis
Streptococcus_salivarius
Streptococcus_sanguinis
Streptococcus_vestibularis
unclassified
LLD
IBS
IBD
PPI
Non_user
User

Drug use is associated to different resistome profiles

In vitro evidence is becoming available that indicates it is not only antibiotic use that can increase antibiotic resistance (AR): non-antibiotic drugs can also contribute to this mechanism[11].

First, we analysed whether the total count of AR genes was increased in users of individual drugs compared to those not using any drugs for all three cohorts separately. For the general population cohort, the total amount of AR genes was increased for eight individual drugs ($p < 0.05$): anti-androgen oral contraceptives, beta-sympathomimetic inhalers, laxatives, metformin, NSAIDs, other oral anti-diabetics, PPIs and triptans. In the IBS cohort, this was the case for two individual drugs: steroid nose spray and calcium channel blockers (p=0.026 and p=0.034, respectively). In the IBD cohort, this increase was present in users of nine individual drugs ($p < 0.05$): PPIs, vitamin D, oral contraceptives, calcium, folic acid, insulin, metformin, opiates and tricyclic antidepressants. Users of metformin showed an increase in AR genes in the general population cohort and IBD cohort (p=0.002 and p=0.046). In both the general population cohort and the IBD cohort, an increase in AR genes was present in participants using PPIs (p=0.001 and p=0.021, respectively) (Supplementary Table 54).

To identify which drug groups were related with an increase of individual AR genes, we analysed the abundances of individual AR genes. In all three cohorts, we identified consistent increases in three AR gene markers in the PPI users compared to participants not using any drugs (FDR < 0.05). These genes belong to *tetA*, *tetB* and *Mel* (ARO:3004033, ARO:3004032 and ARO:3000616, respectively) and are parts of efflux pumps which pump certain types of antibiotics out of the bacteria and thereby inhibit the working mechanisms of these antibiotics[24,25]. For *tetA* and *tetB* this affects the antibiotic group tetracyclines and for *Mel* it affects the antibiotic group macrolides[24,25]. These AR markers have the highest correlations with *Streptococcus parasanguinis* (rho's between 0.56-0.75). We identified three ARs that have also been tested *in vitro*[11]. The AR *TolC*, for example, is known to be involved in multiple multi-drug efflux pumps[24] and was statistically significantly increased in sex drug groups: three in the general population cohort (PPI, statin and metformin) and two in the IBS cohort (steroid nose spray and levothyroxin) and one in the IBD cohort (tricyclic antidepressants). Another example of an *in vitro* tested AR is *mdtP*, also a multi-drug resistance efflux pump, which was increased in metformin users in our general population cohort (FDR=0.001) and in tricyclic antidepressants in the IBD cohort (FDR=0.017, Supplementary Table 55).

Discussion

In this study we have shown the influence of commonly used drugs on gut microbiome composition, microbial functions and AR mechanisms in both the general population and in individuals with GI diseases while also considering the clinical context, where polypharmacy and comorbidities play an important role. In addition, we observed how drug-associated changes also have implications for the clinically relevant feature of AR. Interestingly, for 17 different drugs we observed across the three cohorts, an increase in total AR genes in users compared to participants not using any drugs.

We observed that the overall composition of the gut ecosystem is only consistently altered by the use of PPIs and the number of drugs used. While the effect of PPI use can potentially be explained by changes in acidity facilitating the growth of upper intestinal bacterial in the gut, the effect of the number of drugs used could reflect either severe health conditions that impact the microbiome composition or a bigger stress on the gut environment caused by multiple drug intake. In addition, we did not observe any change in the microbial richness with multi-drug use, suggesting that there is not a clear depletion or colonization of bacteria.

We identified over 500 drug combinations. Even though we could not analyse specific drug combinations separately because of the limited numbers of each combination, we could show the importance of taking multiple drug use into account using two strategies: identifying associations of drug use with individual taxa by including (multivariate) or not including (univariate) all other drug used in our linear models. As depicted in Figure 1, we identified large differences in the number of associated taxa and pathways and the number of different drugs in the univariate and multivariate strategies. This demonstrates the added value of studying these interactions in patient groups in which polypharmacy and comorbidities are common.

In the multivariate meta-analysis, we identified that usage of PPI, laxatives and antibiotics had the largest effect on the gut microbiome composition. These three medication categories have different targets: antibiotics directly target bacteria by inhibiting bacterial growth, and laxatives and proton-pump inhibitors have an impact on the host. A recent study, however, has demonstrated that chemical compounds present in common medication can exhibit inhibitory effect on bacterial species[11]. In the case of proton-pump inhibitors, the impact on the gut microbial composition has been suggested to be consequence of the combination of two mechanisms: indirect impact mediated by the changes in the intestinal pH, promoting the growth of typically oral bacteria, and a direct effect via the inhibition of certain commensal gut bacteria, including *Dorea* and *Ruminococcus species*[7,11,26].

In our cohort a total of 30 participants were using, or had used, antibiotics 3 months previous to fecal sampling. Despite the limited number of users, we showed a decreased relative abundance of *Bifidobacterium* species in the general population, consistent with what has been described previously[4]. The decrease of *Bifidobacterium* abundances has also been shown in in-vitro studies, where multiple antibiotic chemical components impact the growth of these bacteria[11].

A confounding factor in the study of the interaction between laxatives and gut microbiota is a difference in the intestinal transit time in patients using this medication, due to diarrhea or obstipation. For example, increased abundances of *Bacteroides* species have been described in individuals experiencing a fast transit time[27]. This signature, however, has also been observed in mice exposed to the laxative polyethylene glycol (PEG). While there is no evidence

of a direct effect of this chemical compound on the inhibition of bacterial growth, experiments in mice suggest that microbial changes are indirect consequences of the disruption of the gut osmolality[28]. These changes seem to persist even weeks after the PEG administration. However, the long-term effect in humans has not yet been described.

In addition, we found that the use of laxative use was associated with a higher relative abundance of *Alistipes* genus. Interestingly, this genus has also been shown to be decreased in children with chronic functional constipation and to be resistant to bile acids[29], while another identified the deficiency of bile acids in a subset of patients with IBS of the subtype with constipation[30]. These results suggest a role for *Alistipes* in the pathogenesis of constipation.

We also identified an increase of *Methanobrevibacter smithii* in oral steroid users. Interestingly, this species has been associated with obesity and an increase in BMI in both rats and humans [31,32]. Pathways involved in methanogenesis were also increased in oral steroid users, however, these pathways were linked to the abundance of *M.smithii*, therefore, these functional changes are probably consequence of the increased abundance of this archaeon. It is believed that the methane produced by these species facilitates the digestion of polyfructose and thereby plays a role in caloric harvest[31,32]. This could potentially explain the weight gain that is frequently observed in oral steroid users[33]. In our study this effect was evident in the cohort of IBD patients were the larger number of oral steroid users occurred.

Species belonging to the oral microbiome, like *Streptococcus parasanguinis*, are especially characteristic of the gut microbiome of PPI users, which is in agreement with a previous published study[34]. As a consequence of this increase, specific AR mechanisms such as macrolide resistance also appear to be more abundant in faecal samples from PPI users. Previous studies have shown a synergistic effect of macrolides and PPIs, as indicated by the increased success rate of eradication therapy for *H. pylori* in patients receiving macrolides and PPIs versus macrolides alone, and this effect does not appear to be pH-dependent *in vitro*. The macrolide clarithromycin also inhibits the metabolism of the PPI omeprazole[35,36]. Moreover, we also observe an increase of microbial functions characteristic of the oral bacteria, such as carbohydrate degradation pathways and an increase in pathways involved in L-arginine biosynthesis. Interestingly, one previous study has shown an important role for L-arginine in the bioavailability of the PPI omeprazole, which works by increasing the stability and solubility of omeprazole[37].

Our results showed an important role for *Escherichia coli* species in the gut microbiota of metformin users. Even though we could not identify any taxa associated with metformin use, we did identify an increased predicted metabolic potential of this species. Two recent studies exploring the impact of metformin on the gut microbiota showed significant changes in the bacterial composition and metabolic potential[9,10]. Although both studies identify a significant enrichment of *Escherichia coli* in the faecal samples of metformin users, direct causality could not be stablished in *in-vitro* experiments. In our meta-analysis, this trend was also observed. However, it did not reach the significance after multiple testing correction. This could be partially explained by the fact that this species is already enriched in the faecal microbiota of patients with IBD. Furthermore, the metabolic potential of the microbial ecosystem was altered in metformin users. Consistently with previous studies, changes were observed in the lipopolysaccharide and carbohydrates metabolisms. More detailed analyses showed an en-

richment in *E.coli* annotated pathways and gene-families, however, this could partially be due the overrepresentation of this specie in the current databases. Overall, our results support the hypothesis that metformin has an indirect effect on the gut microbiota mediated by changes in the gut environment. Moreover, we replicated the in vitro finding that the AR protein *emrE* was increased in metformin users in the general population (FDR=0.011)[11], indicating that non-antibiotic drugs can also influence the resistome profiles.

Although an interaction between acetaminophen and the gut microbiota has been described[38], we could not replicate this association in our study. In line with our results, the *in-vitro* study of Maier et al. showed that the administration of acetaminophen did not have a negative impact on bacterial growth of 40 common gut species[11]. Therefore, the inclusion of metabolomic measurements together with host genetics is needed in order to identify indirect effects of the microbe-drug interactions.

The complex interaction between the use of medication, the gut microbiota and confounding factor, poses several limitations in our study. Firstly, the cross-sectional nature of this study cannot identify causality in the observed associations. Second, the use of medication by itself is indicative of changes in the health condition of the host, that may also be accompanied by changes in lifestyle, which are both known to influence the microbiome composition in the gut. Third, due to the wide range of disorders that the commonly used medications described in this manuscript are used for, it is difficult to establish a direct relation between medication use and its confounders. For example, PPIs are indicated for treating gastroesophageal reflux (GERD), but are also prescribed for disorders like bloating or co-administered with NSAIDs to prevent ulcers. Moreover, for drugs sold over-the-counter the indication is usually unclear. On the other hand, when drugs are commonly prescribed for a unique indication, such as metformin for type-2 diabetes, it becomes difficult to distinguish between disease impact on the gut microbiota and the effect of the medication use. Fourth, patients using multiple different drugs could be less healthy. Ideally, prospective studies with metagenomes from stool samples are needed at multiple time points, before and after start of certain drugs, to pinpoint the causality of our observed associations. To disentangle these complex relations, the combination of longitudinal studies (from pre-treatment to wash-out period) with in-vitro experiments can be a good approach.

Metagenomic sequencing studies provide insight into the associations between the use of medication and the changes in the microbial population in the gut, which can explain pharmacological mechanisms and side effects. The integration of multiple host and microbial measurements, however, is needed to completely understand the complexity of the pharmacomicrobiomics interactions. For example, faecal metatranscriptomics experiments will bring a better understanding of bacterial dynamics and its functional implications, while metabolic profiling can reveal important host-microbiota interactions affecting the drug metabolisms.

Despite these limitations, we show that our study of microbiome and medication use results in consistent associations between functions and composition of the fecal microbiome and the intake of medication. We further show that the use of multiple drugs is associated with overall gut microbiome composition, either as a result of the drugs themselves or as a proxy for the underlying diseases. It is therefore worth correcting for multiple drug use in future gut microbiome studies in both general population and in GI disease cohorts.

Together our results contribute to the current knowledge of drug-microbiome interactions in a clinical context and provide the basis for further investigations of pharmacomicrobiomics and potential gut-microbiota-driven side-effects of drugs that are currently being prescribed.

Methods

Cohort information

For this study we used three independent Dutch cohorts: 1) a general population cohort, LifeLines-DEEP, consisting of 1539 individuals; 2) 544 patients with IBD from the 1000IBD cohort of the University Medical Center of Groningen (UMCG); and 3) an IBS case-control cohort with 313 participants from Maastricht University Medical Center+ (MUMC+)[39–41]. Drug usage was retrieved from questionnaires in the population cohort and from medical records in the IBD and IBS cohorts. Each medication was classified into categories based on its indication following the Anatomical Therapeutic Chemical code (ATC-code) database and its working mechanism reviewed by medical doctors (Supplementary Table 1).

Faecal sampling collection and metagenomic profiling

Faecal sample collection and processing was performed similarly for all three cohorts. After quality control of the sequenced reads, the microbial taxonomic and functional profiles were determined using *MetaPhlAn2* (v 2.2)[42] and *HUMAnN2* (v 0.10.0)[43], respectively. The Uniref90 and Chocophlan databases were used as a reference for microbial gene identification. Resistome characterisation was performed using *ShortBRED*[44] and the pre-calculated antibiotics database provided with the tool (accession July 2017).

Filtering and diversity measurements

In the IBD cohort, 67 patients with stoma, pouches or short bowel syndrome were excluded. Furthermore, samples with a sequencing depth < 10 million reads were removed (n=30, 22 samples from the IBD cohort and 8 samples from the Maastricht IBS cohort). After filtering, 1883 samples remained for the analyses.

Microbial diversities and dissimilarities were computed using taxonomical *end-points* defined as the lower and non-redundant taxonomical level for each branch of the phylogenetic tree. Bray-Curtis distances and Shannon index were calculated using the functions *vegdist (method = "bray")* and *diversity (index = "shannon")* implemented in the R package *vegan* (v. 2.4-1). Microbial taxa were removed if they were redundant, absent in at

least 90% of the samples in each cohort or if the mean abundance of a taxon was < 0.01%. The remaining taxa values were normalized using the square root arcsine transformation. Microbial pathways were transformed to relative abundance and considered for analyses if they were present in > 10% of the samples in each cohort. Finally, pathway abundances were log transformed keeping the zero values. In total, 194 taxonomical end-points and 321 pathways were evaluated.

Taxonomic contribution to metabolic pathways

Pathways that were shown to be associated with medication use in the multivariate meta-analysis were further investigated. To estimate the bacterial contribution to each pathway we calculated the species-level stratified abundances using the HUmann2 pipeline. Gene families were also extracted using the *humann2_unpack_pathways* script. Values were transformed to relative abundance and log-transformed as described above. For each medication category associated with changes in the metabolic potential of the gut microbiota, the differential abundances in the stratified pathways and gene families were tested using the Wilcoxon signed-rank test. Significant levels were adjusted for multiple testing applying the Benjamini-Hochberg correction.

Statistical analyses

Associations to microbial community measurements

The association between each drug and bacterial diversity (Shannon Index) was evaluated by performing wilcoxon signed-rank tests between users and non-users. The impact of medication categories on the microbial overall composition (Bray Curtis dissimilarities) were estimated using a PERMANOVA test with 10000 permutations as implemented in the adonis function of vegan R package. In addition, the association between number of administered drugs per participant, microbial diversity and composition were tested. Significance levels were adjusted for multiple testing with Benjamini Hochberg method.

Individual cohort associations

Drug associations with microbial features were initially evaluated per cohort using linear models. Due to the multiple medication combinations it was not possible to estimate the effect of drug co-administration, however, to correct for this potential effect, two models were constructed:

(i) Association between individual taxa or pathways and specific drug types, adjusting for the general host factors: age, sex, BMI and sequencing depth.

(ii) Association between individual taxa or pathways and specific drug types adjusted for host factors (age, sex, BMI and sequencing depth) and the effect of the other 40 drugs available in our metadata. Additional covariates were diagnosis (Crohn's disease,

ulcerative colitis or inflammatory bowel disease type unclassified) in the IBD cohort and an IBS diagnosis in the Maastricht IBS cohort and in the general population.

Resistome analysis

Antibiotic resistance (AR) gene abundances were calculated as the mean value of the normalized read counts of each marker representing a gene. AR genes present in < 10% of the participants were excluded from further analyses. Drug users were compared to participants not using any drugs in each cohort separately by performing a Wilcoxon test.

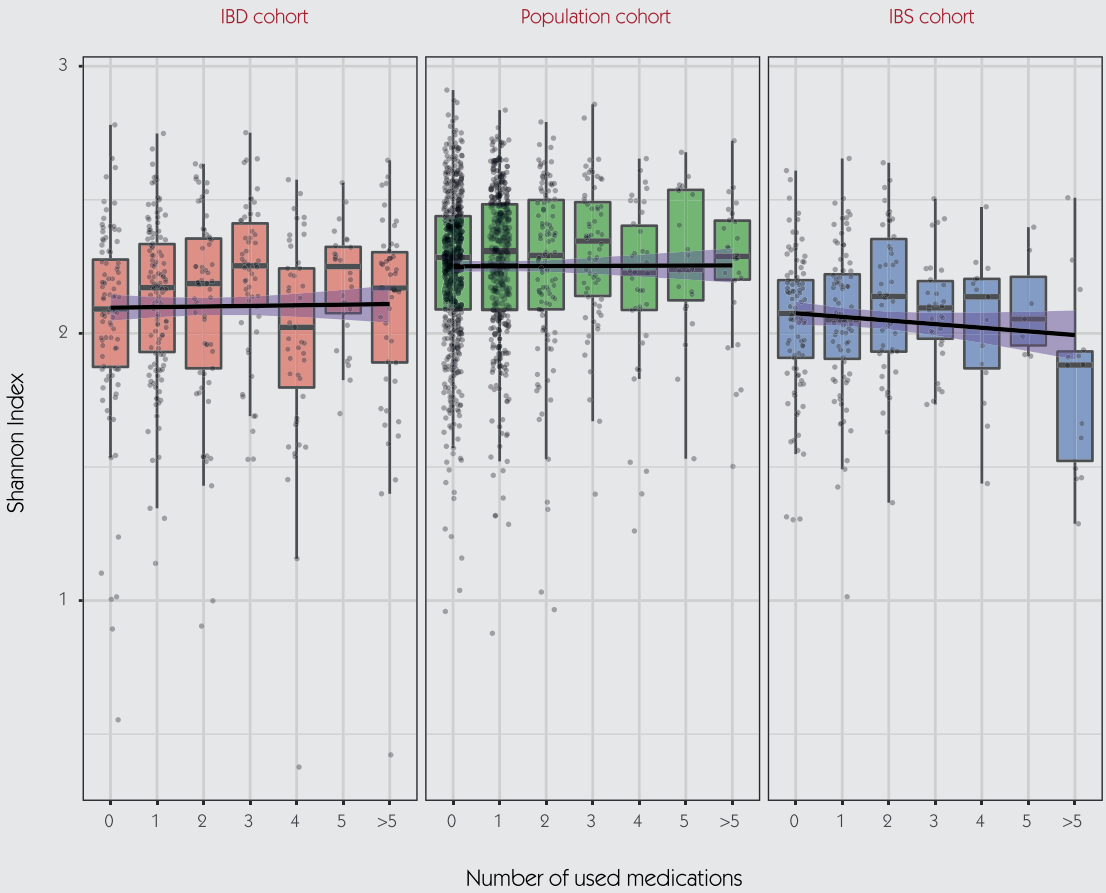
Meta-analysis

Individual cohort associations were combined in a meta-analysis if the specific drug was present in all three cohorts. Overall z-scores and p-values were calculated using an inverse-variance-based approach as described in Willer et al.[45]. The p-values obtained were further adjusted for multiple testing using the Benjamini-Hochberg calculation implemented in the *p.adjust* function in R. Associations with an FDR < 0.1 were tested for heterogeneity using the function *metagen* of the meta R package (v. 4.8-4). Finally, associations were considered to be significant if the meta-analysis multiple testing adjusted p-values were < 0.05 (FDR < 0.05) and the heterogeneity p-value > 0.05.

Individual medication and dosage-dependent effects

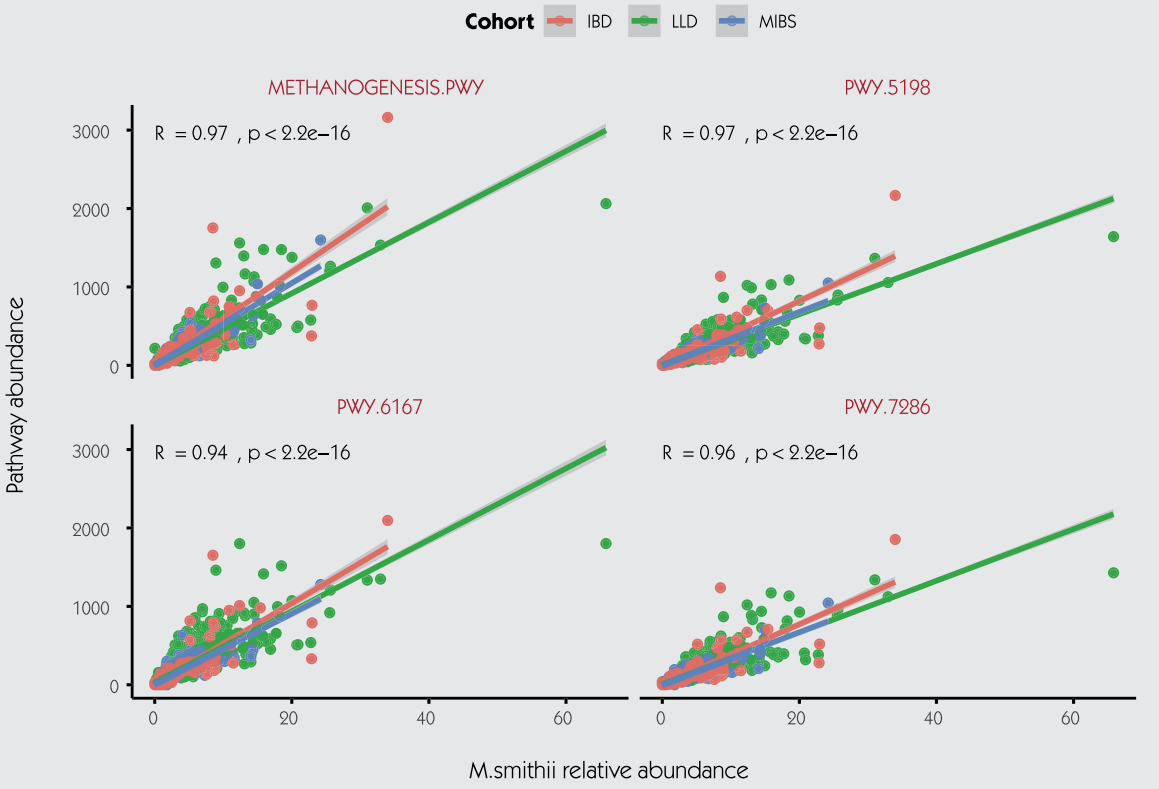
Statistically significant medication-microbiome associations were further assessed for the differential influence of drug types within the same category and the prescription dosages. Medication subtypes were analysed if they were present in at least 5 participants. To evaluate the effect of each medication subtype, the abundance of the associated microbial features was compared between users of a drug subtype and participants not using drugs belonging to the same category. An example of that is the comparison between tetracycline users to participants not using antibiotics. Due to the distribution of the data referring to medication doses (Suppl. table 12), samples were grouped into two categories: "high dose" and "low dose" of each particular drug. For PPI's this threshold was set to a minimum of 40 mg/day for the high dosage group and for metformin this minimum was set at 1000mg/day. Users of laxatives, alpha-blockers, SSRI-antidepressants or antibiotics of our cohort, reported similar prescription patterns or the subtypes within this medication categories showed major differences in dosages. Therefore, we were unable to analyse dosages in these medication categories. Differences between groups were tested using non-parametric t-test (Wilcoxon-test).

Supplementary Figure 1.



Distribution of microbial diversity (Shannon index) stratified per number of medications used for each participant.

Supplementary Figure 2.



Correlation between the relative abundance of Methanobrevibacter smithii and the pathways associated to oral steroids.

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Code availability

Code use for generating the microbial profiles are publicly available at:
https://github.com/WeersmaLabIBD/Microbiome/blob/master/Protocol_metagenomic_pipeline.md

For resistome profiles:
https://github.com/WeersmaLabIBD/Microbiome/blob/master/Protocol_antibiotic_resistance_genes_ShortBred.md

Code used for the statistical analyses is publicly available at:
https://github.com/GRONINGEN-MICROBIOME-CENTRE/Groningen-Microbiome/tree/master/Projects/Medication_metanalysis

Data availability

The raw metagenomics sequencing reads are available for all three cohorts under request in the European Genome-phenome Archive (EGA: https://ega-archive.org). The accession number of the 1000IBD cohort is EGAD00001004194, of the LifeLinesDEEP cohort is EGAD00001001991 and for the MIBS is EGAD00001002668.
The source data underlying Figs 1 and 2 and Supplementary Figs 1 and 2 are provided as a Source Data file.
Supplementary tables and figures can be found:
https://github.com/ArnauVich/Supplementary_tables/raw/master/C8.tar.gz

Writing Assistance

English and scientific editing services were provided by Kate Mc Intyre, PhD, who is employed by the Department of Genetics, University Medical Center Groningen.

Patient consent

Informed consent forms were available for all participants and all were 18 years or older at time of faecal sampling.

Ethical approval

Institutional ethics review board (IRB) approval was available for all three cohorts. Both the Lifelines DEEP and UMCG IBD cohort were approved by the UMCG IRB (ref. M12.113965 and IRB-number 2008.338, respectively). The Maastricht IBS cohort was approved by the MUMC+ IRB (ref. MEC 08-2-066.7/pl).

Authors' contributions

RKW designed the study. AVV, VC, FI and ZM gathered and prepared the data. AVV and VC analysed the data. AVV and VC wrote the manuscript. SS, FI, TS, ZM, DJ, AAMM, JF, CW, AZ and RKW were involved in sample collection, data generation and critically reviewing the manuscript.